EP03/10048

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1.	Your reference	4-32578P2-NFI 8023	
2.	Patent application number *(The Patent Office will fill in this part)*	0300591.5	10 JAN 2003
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	NOVARTIS AG LICHTSTRASSE 35 4056 BASEL SWITZERLAND	
	Patent ADP number *(if you know it)*		
	If the applicant is a corporate body, give the country/state of its incorporation	SWITZERLAND	
4.	Title of invention	Organic compounds	
5.	Name of your agent *(If you have one)*		
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	B.A. YORKE & CO. CHARTERED PATENT AGENTS COOMB HOUSE, 7 ST. JOHN'S ROAD ISLEWORTH MIDDLESEX TW7 6NH	
	Patents ADP number *(if you know it)*	1800001	
6.	If you are declaring priority from one ore more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)* / Date of filing (day/month/year)
7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing (day/month/year)
8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *(see note (d))*	Yes	

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Claim(s)	2 ✓
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Priority documents	
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	One ✓
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application

Signature: B. A. Yorke & Co. — Date: 10 January 2003

12. Name and daytime telephone number of person to contact in the United Kingdom: **Mrs. J. Crook** **020 8560 5847**

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Organic Compounds

The present invention relates to organic compounds, e.g. to an assay for identifying an agent that modulates the activity of a PAK kinase.

In conventional ELISA systems in general either the substance, e.g. an antigen which is to be detected or a specific antibody which binds to the respective antigen, is bound to a solid phase (e.g. microtiter plate) by hydrophobic interactions: the protein interacts with the solid phase, usually a polystyrene surface, at high pH. Although this bond is responsible for all the consecutive steps in the procedure, it remains the weakest bridge to the assay support, the ELISA plate. Strong detergents at higher concentrations such as 0.05% are able to diminish the amount of reagent bound to the plate and can even abolish binding totally. Consecutive steps in ELISA technique, such as e.g. the binding of an antigen to the solid phase-bound antibody and further binding of a second antibody, occur with an affinity of approximately 10^{-12} to 10^{-10} mol per liter. One may view this as being similar to an inverse binding cascade from the bottom of the plate to the top, comparing with a pyramide standing on the top. Another concern is the intramolecular event upon binding. A protein such as a cell-receptor, an enzyme or an antibody behaves very flexible according to its polypeptide structure, which forms a complex architecture in solution. This explains its high specificity and selectivity to the ligands to which they bind in vivo. Enzymatic activities for example may be entirely dependent upon the proper formation of the active site pocket, which itself remains flexible in order to engulf the substrate and release the product.

Most proteins when binding to a given surface react with a dramatic change of their tertiary structure, i.e. they unfold, refold, hide their active site or change their conformation in such a way that their activity towards a given ligand may be altered or even cancelled. In order to circumvent this disadvantage, in conventional ELISA systems a catching antibody is generally used. Said antibody binds to the polystyrene plate and exposes the high affinity hyper-variable region towards the incomig antigen. Said antigen is then detected by a second antibody, which is labelled directly or indirectly (e.g. via biotin/avidin) with an enzyme. This enzyme is able to cleave a chromogenic substrate, which itself is converted from the leucoform to the chromoform and thus visualizes the presence of the antigen in question. But even catching antibodies may affect a given protein and its conformation may be changed. This is demonstrated by many examples of therapeutic antibodies whose mode of action is the blocking of an active site on, or the alteration of a biospecific molecule.

We have now found that structural influences or sterical hindrance by the solid phase may be avoided and all reaction partners are present in defined molar concentration and therefore determination of reaction products may be highly accurate

In one aspect the present invention provides a method for identifying an agent that modulates the activity of a PAK kinase comprising

a) providing a peptide comprising the amino acid sequence S-S-L-R-A-S-T, preferably a labeled peptide, e.g. a biotinylated peptide, in a defined amount,

b) providing a PAK kinase in a defined amount,

c) providing a phosphate containing donor, pref. ATP,

d) contacting the components of a), b) and c) in the absence and in the presence of a candidate compound which is expected to modulate the activity of a PAK kinase for a sufficient period of time so that a reaction mixture is formed and reacting for a pre-determined period of time,

e) transferring at least an aliquot of the reaction mixture formed in d) to a solid phase, which is able to bind to the peptide of a), preferably a solid phase coated with a substrate which is able to bind to the label of the peptide, e.g. a streptavidin-coated solid phase for a biotinylated peptide,

f) determining the amount of phosphorylated peptide bound to the solid phase of e) and determining whether there is a difference in this amount in case a candidate compound was present or absent in the reaction mixture, and

g) choosing an agent that modulates the activity of a PAK kinase.

The principle of this method or assay is as follows: One of the characteristics of a PAK kinase is that this enzyme is able to transfer phosphate from a phosphate containing donor, e.g. ATP, to a substrate comprising a serine and/or threonine amino acid as an acceptor. Such a substrate can be a peptide as provided in a), which is preferably pre-labeled. After a defined contact (incubation) time of the PAK kinase, the substrate, e.g. a peptide according to a), and a phosphate containing donor, e.g. ATP, under defined reaction conditions, like e.g. temperature, pH, salt concentration etc., the reaction is stopped and an aliquot of the reaction mixture is transferred to a solid phase, e.g. a streptavidin-coated microtiter plate, where the preferably pre-labeled, more preferably biotinylated, and to a measureable extent the phosphorylated peptide is trapped and the degree (amount) of phosphorylation is

quantified by means of an appropriate antibody against a phosphoserine, a phospho-threonine or both amino acids on a phosphoserine-threonine containing peptide. Said antibody can bear a label, e.g. horse radish peroxidase (=POD) or can be detected with a second antibody which specifically recognizes the first antibody and which is labeled, e.g. enzyme-labeled or fluorescent labeled.

During the whole reaction of the kinase with its reaction partners all molecules are able to float freely in solution and thus are not influenced by the structural modifications of solid phases. They receive their conformation by the given pH and salt concentration and exert their maximum binding or reaction affinity. Also the exact amounts of all the reaction components are known. After a given contact time, selected according to the results of appropriate equilibrium measurements, the phosphorylated serine and/or threonine containing peptide formed has to be deprived of all other components. The peptide therefore is labeled, e.g. biotinylated prior to the above reaction, and after stopping the reaction an aliquot of the reaction mixture is transferred to a solid phase, preferably to a streptavidin-coated microtiter plate.

The solid phase is preferably a plastic plate like a polystyrene or polyvinyl plate, esp. a microtiter plate. Also microbeads can be used as a solid phase, preferably coated microbeads. The coating used for the solid phase depends e.g. on the label used for the peptide. The material of the coating should be able to form a complex with the label used for the peptide, e.g. the material used for the coating can be streptavidin which is covalently bound to a solid support and the label used for the peptide can be biotin.

In case a PAK kinase activity modulating substance is present in the sample the phosphate transfer reaction (=phosphorylation) does not occur or occurs only to a limited/reduced extent. Since the reaction takes place in solution at well-defined molar concentrations of all the essential components used in this assay, and at defined temperature, pH and salt conditions, structural influence of solid phases are perferctly eliminated in this assay.

A candidate compound includes compound(s)(libraries) from which its influence on the PAK kinase can be determined. Compound (libraries) include for example oligopeptides, polypeptides, proteins, antibodies, mimetics, small molecules, e.g. low molecular weight compounds (LMW`s).

An agent is a compound which influences (inhibits) the activity of a PAK kinase.

An agent is one of the chosen candidate compounds and may include oligopeptides, polypeptides, proteins, antibodies, mimetics, small molecules, e.g. low molecular weight compounds (LMW`s). An agent includes one or more agents.

In a preferred embodiment of the present invention the binding of e.g. streptavidin with the surface of the solid phase, e.g. a microtiter plate, is perfectly made covalently in order to build up an affinity cascade from the bottom of the solid phase to the top of the reactants. For example, a microtiter plate chemically modified with e.g. a N-oxysuccinimide ester coating is used, which reacts with nucleophiles such as primary amines under formation of a covalent bond. This bond is stronger than 10^{-15} moles per liter. For the next step, the labeling, e.g. biotinylation of the peptide, e.g. a chemically modified biotin, such as NHS-LC-Biotin is used, which comprises an extended spacer arm of approximately 22.4 Å in length. This long chain analogue reduces steric hindrance associated with the binding of four biotinylated molecules on one streptavidin molecule. The target of biotinylation on the peptide is thereby well defined and interference with the ligand is avoided. The binding affinity of e.g. biotin to streptavidin is also known to be 10^{-15} moles per liter. Thereby the first two steps of the binding cascade are established and reach from the bottom of the plate to streptavidin to the biotinylated peptide. The affinity constants are decreasing from the bottom to the top. At this stage the reactants, which have found their partners in solution under defined conditions, are trapped by means of e.g. the streptavidin coated plate and the bound peptide is then detected, e.g. with an appropriate enzyme-labeled antibody, e.g. an POD-labeled antibody against the phosphoserine or phosphothreonine containing peptide or with a tandem antibody system, e.g. as appropriate, such as described earlier.

Some of the advantages of the assay according to the present invention in comparison to standard ELISA systems where the kinase or the peptide will first be attached to the solid support with e.g. either a catching antibody or by high pH (9.6) are the following:

a) The structural influence or sterical hindrance by the supporting solid phase ot the catching antibody in standard sandwich-ELISA may be avoided,
b) no treatment of the kinase or the peptide at alkaline pH,
c) the reaction partners are present in defined molar concentrations,
d) high specificity for a kinase because of the peptide used, and
e) reduced working steps and easy handling by e.g. robotics.

The peptide, serving as a specific substrate for a PAK kinase, e.g. a PAK-2 kinase, contains a serine and/or a threonine motif (= sequence of other amino acids), preferably both amino acids are included in the motif. The peptide is preferably a A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S peptide; preferably it is a labeled peptide, e.g. a biotinylated peptide.

In a preferred embodiment of the present invention the phosphorylated amino acid serine and/or threonine of the peptide bound to the solid phase is detected with an antibody selected from the group consisting of anti-phosphoserine antibody, anti-phosphothreonine antibody and anti-phosphoserine-threonine antibody.

In another aspect the present invention provides a kit for identifying an agent that modulates the activity of a PAK kinase comprising as components

a) a peptide comprising the amino acid sequence S-S-L-R-A-S-T, preferably a labeled peptide,
b) a PAK kinase,
c) a phosphate containing donor, preferably ATP, and
d) an antibody selected from the group consisting of anti-phosphoserine antibody, anti-phosphothreonine antibody and anti-phosphoserine-threonine antibody, and
e) optionally a solid phase, preferably coated with a substrate which is able to bind to the label of the peptide.

The antibody of d) can itself bear a label for detection or can be detected via a second antibody which specifically recognizes the first antibody and is labeled, e.g. enzyme or fluorescent labeled.

The peptide has preferably the sequence A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S and is preferably labeled, e.g. biotinylated.

Said kit may further comprise a substantial component, e.g. including an appropriate environment of a sample to be tested and, e.g. appropriate means to determine the effect of a candidate compound in a sample to be tested.

In another aspect the present invention also provides a method for identifying an agent that modulates the activity of a PAK kinase comprising an assay according to the present invention.

In a further aspect the present invention provides a method for differentiating between an agent that modulates the threonine specific activity of a kinase and an agent that modulates the serine specific activity of a kinase, e.g. a PAK-2 kinase, comprising an assay according to the present invention wherein the peptide bound to the solid phase is detected with an anti-phosphoserine antibody and an anti-phosphothreonine antibody and determining whether there is a difference in the signals determined with each single antibody.

In another aspect the present invention provides the use of a peptide comprising the amino acid sequence S-S-L-R-A-S-T, preferably the sequence A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S, for identifying an agent that modulates the activity of a PAK kinase.

In the following examples all temperatures are in degree centigrade and are uncorrected.

The following ABBREVIATIONS are used:

ATP	adenosine triphosphate
DMF	dimethylformamide
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
mAb	monoclonal antibody
PBS	phosphate-buffered saline
PBS def.	NaCl 8 g/l; KH_2PO_4 0.2 g/l; KCl 0.2 g/l; Na_2HPO_4.2aq 1.44 g/l; pH 7.2
PBST	PBS + 0.05% Tween 20
POD	horse radish peroxidase
RT	room temperature

EXAMPLE: Assay procedure

1. Preparation of covalently–bound streptavidin microtiter plates

To 12 ml of PBSdef. of pH 9, 6 µl of streptavidin solution is added and stirred. 100 µl of this solution are pipetted into each well of a Costar Amine plate, which is taken directly from 4° storage and dismanteled from its protective envelope prior to pipetting. The whole procedure is performed in the dark and the plate must be kept in a light protective aluminium foil at RT. Thereafter the consecutive steps can be done under normal conditions:

Five times washing of the plate with PBST and tapping onto a paper towel in order to free the plate from residual moisture, addition of 360 µl of 0.5 M Tris-Cl pH 8 and incubation at RT. After a five times washing as above, 200 µl of blocking solution is added into each well and kept at RT for 30 minutes. Then the plate is washed once more and stored in a sealed plastic box at 4°.

2. Biotinylation of the peptide

As a substrate a peptide comprising the amino acid sequence A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S is used. The peptide is dissolved in either DMF or DMSO and kept in a light protected vial. A solution of Immunopure NHS-LC-Biotin II (Pierce) is prepared in above solutes and kept under light protection. The biotin solution is added stepwise to the peptide containing vial at RT. The reaction is stopped by addition of 1 M Tris-Cl pH 8 and tumbled. The reaction mixture is subsequently transferred into Eppendorf vials and centrifuged in order to clarify the solution of biotinylated peptide. This supernatant is subjected to reversed phase chromatography or size exclusion chromatography.

3. Key steps of assay procedure

a) Mix biotinylated peptide, ATP, PAK-kinase and optionally a candidate compound

b) Incubate

c) Provide plates with covalently coupled streptavidin (=SA-plates)

d) Transfer aliquot of the reaction mixture formed after step b) onto SA-plate

e) Wash the plate free of unbound substances

f) Add detecting antibody (= POD-linked antiphosphoserine antibody and/or POD-linked antiphosphothreonine antibody or a labeled secondary antobidy recognising the first unlabeled antibody) and incubate

g) Wash and develop enzyme substrate, e.g. peroxidase substrate, and

h) Determine the extinction at defined wavelength, e.g. at 450/690 nm to quantify the enzymatic reaction and thus determine the amount of phosphorylated (phosphorylation degree) serine and/or threonine.

Patent Claims:

1. A method for identifying an agent that modulates the activity of a PAK kinase comprising
a) providing a peptide comprising the amino acid sequence S-S-L-R-A-S-T, preferably a labeled peptide, e.g. a biotinylated peptide, in a defined amount,
b) providing a PAK kinase in a defined amount,
c) providing a phosphate containing donor, pref. ATP,
d) contacting the components of a), b) and c) in the absence and in the presence of a candidate compound which is expected to modulate the activity of a PAK kinase for a sufficient period of time so that a reaction mixture is formed and reacting for a pre-determined period of time,
e) transferring at least an aliquot of the reaction mixture formed in d) to a solid phase, which is able to bind to the peptide of a), preferably a solid phase coated with a substrate which is able to bind to the label of the peptide, e.g. a streptavidin-coated solid phase for a biotinylated peptide,
f) determining the amount of phosphorylated peptide bound to the solid phase of e) and determining whether there is a difference in this amount in case a candidate compound was present or absent in the reaction mixture, and
g) choosing an agent that modulates the activity of a PAK kinase.

2. A method according to claim 1 wherein the peptide has the sequence A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S and is preferably biotinylated.

3. A method according to any one of claims 1 or 2 wherein the the phosphoserine and/or phosphothreonine moiety of the bound peptide is detected with an antibody selected from the group consisting of anti-phosphoserine antibody, anti-phosphothreonine peptide antibody and anti-phosphoserine-threonine antibody.

4. A kit for identifying an agent that modulates the activity of a PAK kinase comprising as components
a) a peptide comprising the amino acid sequence S-S-L-R-A-S-T, preferably a labeled peptide,
b) a PAK kinase,
c) a phosphate containing donor, preferably ATP,

d) an antibody selected from the group consisting of anti-phosphoserine antibody, anti-phosphothreonine antibody and anti-phosphoserine-threonine antibody, preferably an enzyme-labeled antibody, and

e) optionally a solid phase, preferably coated with a substrate which is able to bind to the label of the peptide.

5. A kit according to claim 4 wherein the peptide has the sequence A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S.

6. A kit according to any one of claim 4 or 5 wheren the peptide is biotinylated.

7. A method for differentiating between an agent that modulates the threonine specific activity of a kinase and an agent that modulates the serine specific activity of a kinase comprising a method according to any one of claims 1 to 3 wherein the bound peptide is detected with an anti-phosphoserine antibody and an anti-phosphothreonine antibody and determining whether there is a difference in the signals determined with each single antibody.

9. Use of a peptide comprising the amino acid sequence S-S-L-R-A-S-T, preferably the sequence A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S, for identifying an agent that modulates the activity of a PAK kinase.

IL/9-Jan-2003

Abstract

The invention relates to organic compounds, e.g. a method for identifying an agent that modulates the activity of a PAK kinase. Also provided is a kit for identifying such an agent.

PCT Application
PCT/EP2003/010048